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Development and Clinical Validation of Monoclonal Antibodies Targeting Plasmodium LDH for Enhanced Malaria Diagnostics

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ABSTRACT

Malaria diagnostics alternately require tools that are highly sensitive, species-specific, and operationally practical, particularly in resource-limited settings. In this study, the development and characterization of novel monoclonal antibodies (mAbs) targeting native *Plasmodium* lactate dehydrogenase (pLDH), a key glycolytic enzyme was expressed during active infection. BALB/c mice immunized with purified native pLDH generated moderate-titer antisera (e+1:6400), leading to the selection of six hybridoma clones secreting IgG1/IgG2b mAbs with no cross-reactivity to human LDH. Epitope binning identified two non-overlapping mAbs, 3D3C4 and 3G10G9, which were integrated into a sandwich ELISA with a limit of detection of 0.078 µg/ml. Clinical evaluation using 54 *Plasmodium falciparum* positive samples and 281 endemic controls demonstrated 100% sensitivity and 99.6% specificity. This pLDH-targeted ELISA addressed critical limitations in current malaria diagnostics by enabling accurate detection of active *P. falciparum* infections and offering potential for therapeutic monitoring. Its compatibility with molecular methods and adaptability to point-of-care platforms make it a promising tool for malaria elimination initiatives. Ongoing work aims at expanding species specificity and validating performance across diverse endemic settings.

Key words: Malaria screening, antibody production, plasmodium lactate dehydrogenase (pldh), monoclonal antibodies

INTRODUCTION

Globally, an estimated 2.2 billion malaria cases and 12.7 million malaria-related deaths were averted between 2000 and 2022. Most of these were in the WHO African Region (82% of cases, 94% of deaths), followed by the WHO South-East Asia Region (10% of cases, 3% of deaths; WHO Malaria Report, 2023). Despite these gains, malaria remains a major public health challenge, and rapid, accurate laboratory diagnosis is essential for effective treatment.

Currently, the primary method for diagnosing malaria is microscopic examination of blood smears. However, this approach is time-consuming and requires trained personnel, which can be a major limitation in remote or resource-limited settings. While polymerase chain reaction (PCR) offers a highly sensitive alternative, its use is limited by high cost and restricted availability in routine clinical settings.

In recent years, rapid diagnostic tests (RDTs) have emerged as a quicker and more affordable alternative. RDTs are widely used in many malaria-endemic areas and require minimal training (Martiáñez-Vendrell *et al.,* 2022). Nonetheless, the limited analytical sensitivity of pLDH-based RDTs poses challenges for accurate diagnosis in low-parasitemia infections, particularly in remote areas. Despite WHO recommendations favouring RDTs when microscopy is unavailable, access to reliable diagnostics remains uneven. Most commercially available malaria RDTs detect either *Plasmodium falciparum* histidine-rich protein 2 (HRP2) or *Plasmodium* lactate dehydrogenase (pLDH; Coldiron *et al.*, 2019). The preferred first-line diagnostic method varies by country in South-east Asia, as does the type of RDT employed. Some tests target antigens such as pLDH or HRP2, and the choice of diagnostic tool often depends on local epidemiological and logistical considerations. For instance, Malaysia predominantly relies

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on microscopy, whereas certain regions in Indonesia utilize RDTs more frequently (Tan *et al.*, 2022).

Unlike pfHRP2, which is susceptible to genetic variation and gene deletions, pLDH is a well-conserved antigen across *Plasmodium* species. However, the diagnostic utility of pLDH-based RDTs for detecting *P. knowlesi* infections remains underexplored. Notably, the most recent round of the WHO *Malaria RDT Evaluation Programme* (2018) showed significantly improved performance, with the best pLDH-based tests achieving nearly 100% detection of *P. falciparum* and *P. vivax* at parasite densities as low as 200 parasites/ìl (World Health Organization, 2018).

Comparative studies suggest no significant differences in performance between HRP2- and pLDH-based RDTs for malaria detection, both of which use lateral flow immunochromatographic assays to identify target antigens (Orish *et al.,* 2018). However, the efficacy of HRP2 and LDH as diagnostic targets remains debated, partly due to regional genetic variations that can reduce test sensitivity (Costa *et al.,* 2021; Kaaya *et al.,* 2022). Although the use of pLDH-specific monoclonal antibodies in immunochromatographic tests has shown promise, the sensitivity and specificity of commercial kits vary considerably. It is unclear whether this variability stems from the underlying biochemical reaction or from differences in test platform technologies (Coldiron *et al.,* 2019).

Therefore, continued efforts to develop robust and reliable RDTs are essential, particularly those incorporating high-affinity monoclonal antibodies targeting native pLDH. Such improvements may help overcome the challenges posed by genetic diversity and enhance the overall accuracy of malaria diagnosis.

MATERIALS AND METHODS

Following written informed consent, blood samples were collected from healthy individuals in Surat, Gujarat, through blood donation programs organized by the Surat Raktadan Kendra and Research Centre (SRKRC), a regional blood transfusion and NABH-accredited centre. All procedures were followed in accordance with the guidelines issued by the same institution, and the study protocols were approved by the Institutional Ethics Committee of SRKRC (Approval No: SRKRC/RP/02/2021). Samples from both infected and healthy individuals were subsequently obtained from SRKRC, India. Informed consent was obtained from all participants prior to sample collection. Infected samples were confirmed using rapid diagnostic tests (RDTs) and ELISA. Blood was collected in heparinized tubes, centrifuged, and plasma was separated and stored for further testing. A total of 54 Plasmodium falciparum-positive samples were included, comprising 23 microscopy-confirmed and 31 PCR-confirmed cases, while 281 endemic control samples tested negative for malaria (Tan *et al.,* 2022).

For the study, parasite lactate dehydrogenase (pLDH) antigen was procured by purifying native Plasmodium pLDH from infected red blood cells using a previously standardized protocol at PDARC, SRKRC (unpublished data). The purification process distinctly separated human LDH (hLDH) from parasite-derived LDH (pLDH), and the purified pLDH antigen was utilized for immunization and subsequent monoclonal antibody screening. All animal experiments followed approval by the Institutional Animal Ethics Committee of Nagpur Veterinary College, India (Approval No: NVC/IAEC/01/2021). Two BALB/c mice were maintained under controlled conditions for six months, and immunizations were performed at Genext Genomics Pvt. Ltd., Nagpur, Maharashtra, India (Liu *et al.,* 2019).

Two female BALB/c mice, aged 6-8 weeks, were immunized with native pLDH emulsified in Freund's complete adjuvant. They were maintained under a regulated 12-h light-dark cycle at 24 °C with stable humidity, with food and water provided ad libitum. Booster doses were administered as required, and blood samples were collected from the caudal vein. Serum was stored at –20°C, and antibody titers were determined by indirect ELISA using microplates coated with 2 ìg/ml of native pLDH. The serum sample showing the highest antibody titer was selected for hybridoma fusion. Freshly isolated splenic lymphocytes from immunized mice were then fused with SP2/0-Ag14 murine myeloma cells following polyethylene glycol (PEG)-mediated fusion protocols. Hybridoma supernatants from primary clones were screened for reactivity

against native pLDH via indirect ELISA, and positive clones were sub-cloned to ensure monoclonality before expansion. The resulting monoclonal antibodies were subsequently purified and cryopreserved for further characterization and applications (Pohanka *et al.,* 2016; Chou *et al.,* 2022).

The isotypic classification of the monoclonal antibodies was determined using a commercial isotyping kit (Sigma-Aldrich), while purification was performed by loading hybridoma culture supernatants onto a protein A–Sepharose column pre-equilibrated with glycine–NaOH buffer containing NaCl. Bound antibodies were eluted with glycine–HCl buffer, neutralized with Tris–HCl to achieve physiological pH and dialyzed against PBS. Protein concentrations were assessed with a Nanophotometer (Pohanka*,* 2017; Miura *et al.,* 2023). For biotin labelling, purified antibodies (1 mg/ml) were incubated with biotin (2 mg/ml) for 2 h at room temperature, followed by purification on a PD-10 desalting column with PBS elution. Fractions were evaluated spectrophotometrically, and peak antibody fractions were further validated using dot blot assay with streptavidin–HRP conjugate. Positive fractions were pooled and stored at –20°C for subsequent experimentation (Janardhan, 2018; Liu *et al.,* 2019).

To assess reactivity, ELISA protocols were employed with necessary modifications. In indirect ELISA, plates were coated with 500 ng of native *P. falciparum* pLDH and incubated overnight, blocked with non-fat milk, and then exposed to hybridoma supernatants. Detection was performed using an HRP-conjugated rabbit anti-mouse IgG antibody, and absorbance was read at 450 nm (Stat Fax 4200). Sandwich ELISA assays involved coating microtiter plates with 500 ng/ml monoclonal antibodies, blocking with skim milk, and adding serial dilutions of purified native pLDH antigen. After incubation with biotinylated secondary antibodies and streptavidin-HRP, detection was performed using TMB substrate, and combinations demonstrating strong reactivity were identified for capturing circulating native pLDH in patient sera (Jang *et al.,* 2018).

Circulating pLDH antigen in clinical sera was ultimately quantified by sandwich ELISA. Plates were coated with capture antibodies at 0.02 mg/ml, incubated overnight, blocked and exposed to diluted sera (1:50). Detection employed biotinylated monoclonal antibodies as secondary's, followed by streptavidin-HRP and substrate development. Absorbance was measured at 450 nm, and samples were defined as positive if their OD exceeded the mean plus three standard deviations of controls (Yucel and Akcael*,* 2018).

All statistical analyses were conducted using Graph Pad Prism (version 10.0.0, GraphPad Software, San Diego, California, USA). Comparisons between two individual data sets were made using the Mann–Whitney test, and differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The successful purification of Plasmodium lactate dehydrogenase (pLDH) was confirmed by differential cofactor zymography. APAD-dependent activity produced a distinct pLDH band, while NAD-based detection eliminated the possibility of human LDH contamination, validating the purity and specificity of the parasite-derived antigen required for immunization and assay development (Fig. 1).

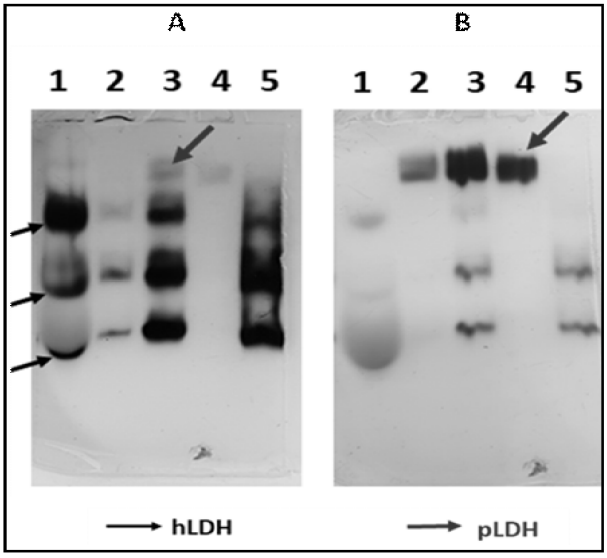

Fig. 1. Purification profile of pLDH in the Native PAGE subjected to (A) Zymogram analysis using MALSTAT reagents containing NAD and (B) - Zymogram analysis using MALSTAT reagents containing APAD.

From 30 hybridoma clones initially generated, six stable monoclonal antibodies (mAbs) were obtained and characterized. These antibodies, predominantly of the IgG1–kappa subclass, demonstrated high specificity for Pf pLDH, with

negligible cross-reactivity to human LDH (A450 d" 0.1; Fig. 2). SDS-PAGE of purified antibodies showed distinct heavy (~55 kDa) and light (~27 kDa) chains, confirming purity and structural integrity. Epitope mapping revealed three non-overlapping binding regions, suggesting complementary recognition useful in capture–detection assays (Fig. 3). Among these, mAbs 3A9D9 and 3D3C3 exhibited the highest affinity, detecting native pLDH at concentrations < 7 ng, while moderate reactivity was observed in 3G10G9 and lower responses in 1B1D7 and 2D3G2 (Fig. 4A, B).

A sandwich ELISA format was optimized by testing multiple antibody combinations. The

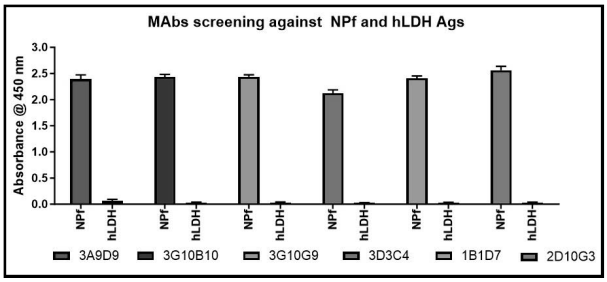

Fig. 2. Native pf/pv pLDH and hLDH (2 ìg/ml) concentrations were coated on ELISA plates and incubated with respective mAbsculture supernatants. The binding of the mAbs to the antigen was determined by incubation with rabbit anti-mouse Ig – HRP conjugate followed by addition of the substrate, TMB and H_2O_2.

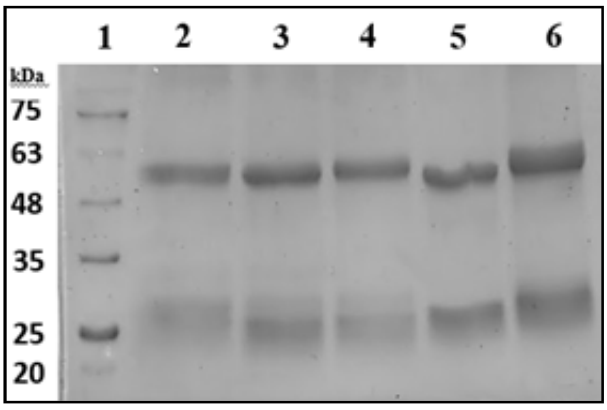

Fig. 3. SDS-PAGE analysis of purified pLDH Antibody (Lane:1-Molecular weight marker, Lane:2–3D3C4, Lane:3–3G10G9, Lane:4–3A9D9, Lane:5–1B1D7, Lane:6-2D3G2) checked by Coomassie Blue staining under reducing condition.

pairing of 3D3C4 as a capture mAb with biotinylated 3G10G9 as the detection antibody showed consistently superior sensitivity (~7 ng of antigen; Fig. 5A, B). and signal-to-noise ratios compared to other tested combinations. This optimized configuration was advanced for clinical sample evaluation.

Validation of the sandwich ELISA using 54 confirmed malaria-positive plasma samples (23 microscopy and 31 PCR confirmed) and 281 endemic malaria-negative controls revealed excellent diagnostic performance. At a cut off of 0.2 ng/ml, the assay achieved 100%

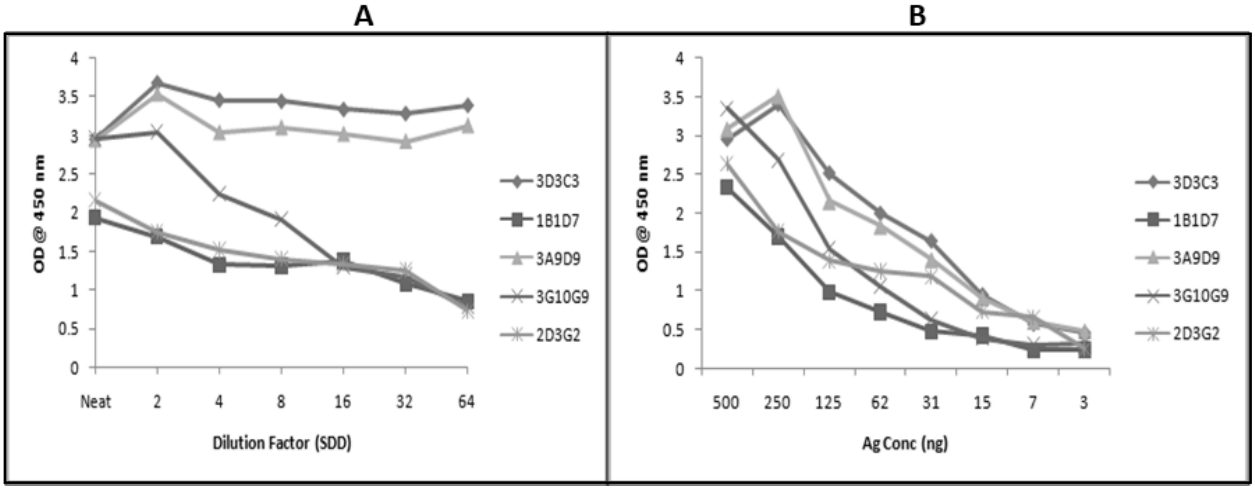

Fig. 4. Reactivity of mAb against native PLDH antigen by ELISA (A) The wells of ELISA plate were coated with antigen concentration (200 ng/well) followed by incubation with serially double diluted (SDD) culture supernatants of the five selected mAbs. (B) The wells of ELISA plates were coated with varying concentrations of native PLDH protein (500 ng–3 ng), followed by incubation with 100 ìl of culture supernatants of the five selected mAbs. The rest of the procedure followed as described in Indirect ELISA (Materials and methods). The results for both the assays expressed as mean ± SD of three independent experiments.

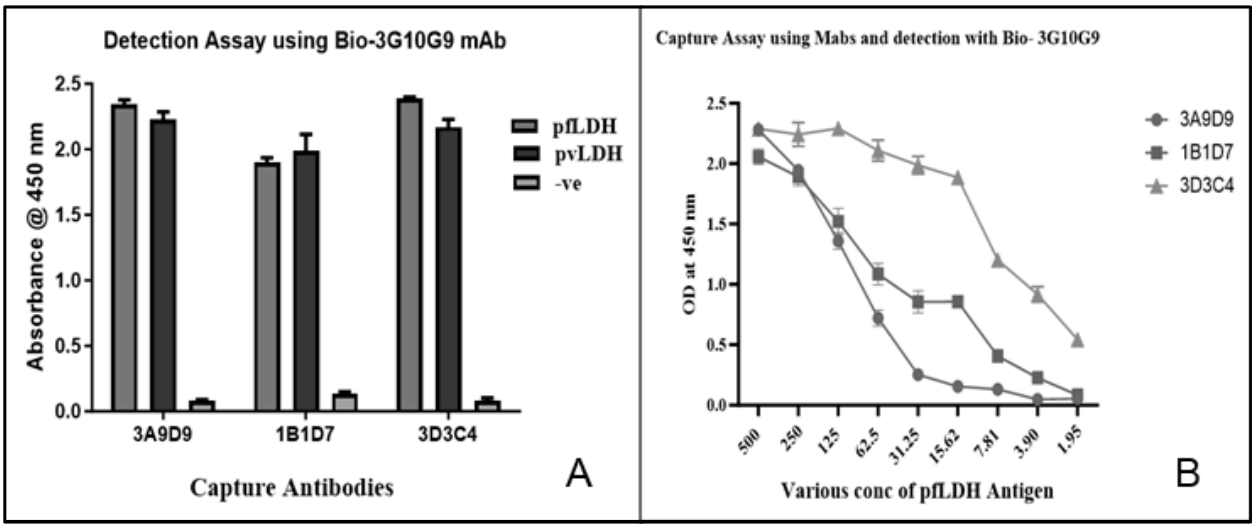

Fig. 5. (A) Capture ELISA to detect native parasite Antigens: Three different combinations of capture and detection antibodies were used to evaluate native pLDH. Capture Abs (0.5 µg/well) were coated in ELISA wells separately and incubated, retaining antigens detected with respective biotinylated 3G10G9 MAb followed by streptavidin-peroxidase and developed. (B) Sandwich assay was performed to detect the various concentrations of pLDH antigens to select best combination of capture and detection antibody.

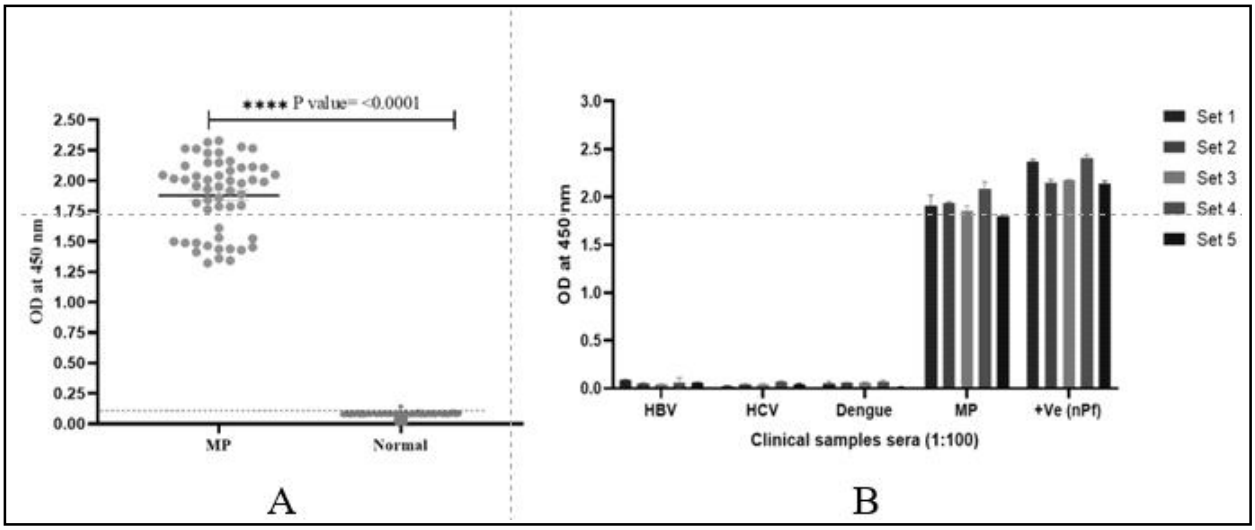

Fig. 6. (A) Capture assay was performed using this combination (3D3C4 capture mAb and bio-3G10G9 as detection) to detect circulating antigens in clinical samples. The mean values of optical density OD±3SD plotted in triplicate to show circulating malarial antigens in clinical samples. (B) Cross-reactivity was evaluated using confirmed positive samples from other infections, including HBV, HCV and Dengue (n = 5 each). No detectable cross-reactivity was observed, confirming assay specificity the assay's diagnostic robustness and translational potential for field-based malaria detection.

sensitivity (detecting all Pf-positive samples) and 100% specificity (no false positives in controls). Statistical analysis confirmed a highly significant difference in mean absorbance values between positive and negative groups (malaria-positive OD = 0.361±0.114 vs. controls = 0.076±0.001, $P < 0.001$). Moreover, the assay demonstrated complete absence of cross-reactivity with sera from patients with other common infections including HBV, HCV and Dengue, underscoring both sensitivity and specificity (Fig. 6A, B).

These results establish that the generated mAbs exhibited not only high specificity for parasite LDH but also strong translational potential. By strategically combining

antibodies with complementary epitope recognition, the sandwich ELISA reached a detection limit that rivaled or exceeded many currently available field-deployed tests. The high accuracy and robustness of the assay were especially relevant for malaria-endemic regions, where rapid, sensitive and affordable diagnostics remain vital for effective case management and control.

Beyond technical performance, the study highlighted broader implications for India and globally. Malaria remains endemic across Asia-Pacific and Africa, with diagnostics estimated to represent a USD 800M–1B global market by 2025, dominated by RDTs. While India currently relies heavily on imports, these findings provide a proof-of-concept for successful indigenous development of diagnostic-grade antibodies and assay platforms. Such initiatives align with the *"Make in India"* program, reducing import dependency while fostering self-reliance in biomedical manufacturing. By demonstrating that high-quality diagnostic antibodies can be produced, purified and validated locally, this work sets a foundation for cost-effective healthcare tools tailored for regional needs.

Looking forward, these antibodies and the validated ELISA format may serve as the basis for the development of scalable, point-of-care rapid assays and their integration with emerging diagnostic platforms, including portable PCR and CRISPR-based tools. Such innovation will not only strengthen India's health security but also position the country as a global supplier of next-generation diagnostics. Investment in local biomanufacturing, synergized with public–private collaborations and sustained R&D funding, will be critical to expand diagnostic capacity and enhance preparedness against malaria and other infectious diseases.

CONCLUSION

This study reports the purification of Plasmodium pLDH, development of highly specific monoclonal antibodies and validation of a sensitive sandwich ELISA assay capable of detecting circulating malaria antigens with 100% sensitivity and specificity. The work underscores both a scientific and strategic achievement-advancing indigenous innovation in infectious disease diagnostics while providing a pathway toward reducing reliance on imports. These findings exemplify how concerted R&D efforts in India can yield cost-effective diagnostic solutions, with implications for improved disease surveillance, patient care, and stronger national and global healthcare resilience.

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